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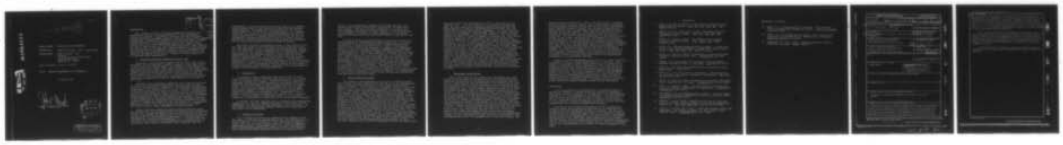

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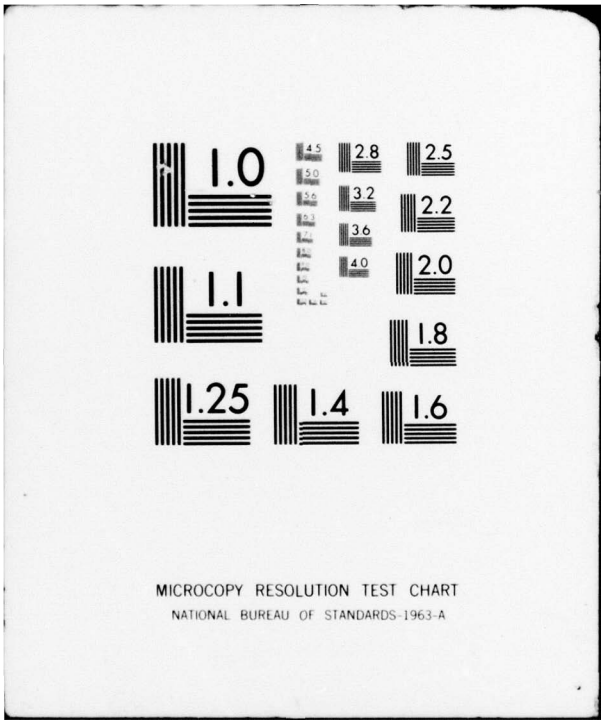
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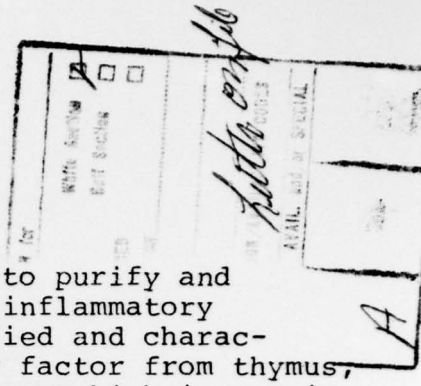

INTRODUCTION

For a number of years I have been attempting to purify and characterize chemically various messengers of the inflammatory process and response. Specifically, we have purified and characterized chemically macrophage migration inhibitory factor from thymus, lymphotactin (a chemotactic substance from the thymus which is specifically chemotactic for lymphocytes, but not other cells) and a permeability factor from transformed lymphocytes, lymphoid tissue and lung. These results will be discussed separately. Most recently, and for the bulk of the past year I have focussed on the purification and characterization of mediators important to the inflammatory response of the lung, particularly, Pulmonary Permeability Factor and Pulmonary Macrophage Growth Factor. Results of these studies will be discussed below sequentially.

1. Macrophage Migration Inhibitory Factor (MIF)

Previously, using contract support from the ONR we had firstly developed a new and extremely sensitive method for the assay for MIF in four hours; the "scratch assay" (1); secondly we had indicated, using this sensitive assay, that aqueous extracts of the thymus from immunologically sensitized guinea pigs or calves (but not unsensitized animals) contained an MIF-like activity which shared all the then known properties of classical MIF (2). We also demonstrated that this activity could be inhibited by epsilonaminocaproic acid derivatives such as amino-valeric acid at concentrations of the order of 10^{-6} M (3).

Interest in this particular lymphokine stems from the fact that it is the first well-defined biological activity produced extracellularly by transforming lymphocytes. Unfortunately, transforming lymphocytes do not produce a lot of it and, therefore, biochemically it has been elusive and difficult to purify. Further, we have found that MIF is extraordinarily subject to proteolysis by contaminating tissue proteases and hence a large amount of the activity is degraded enzymatically during the initial stages of purification. The role of this lymphokine in immunologically mediated inflammation and particularly delayed-type hypersensitivity has been discussed and documented in a number of reviews over the past decade (4). Biologically this activity was often, if not always, co-mingled with another functional activity, namely Macrophage Activation Factor.

Having developed a sensitive assay procedure which was quick and reliable and, having demonstrated that immunologically stimulated animals had a thymus from which could be extracted a MIF activity similar in properties to classical MIF, we then isolated and purified this material to electrophoretic homogeneity at 3 different pH in acrylamide gel analytical electrophoresis (5). Purified thymic MIF was found to be a molecule of approximately 36,500 daltons with an isoelectric point close to neutrality, which contained only two

carbohydrates, one being sialic acid and the other one an unknown neutral hexose which we have tentatively identified as O-methyl-glucopyranoside. We suggest that the inhibition of MIF by very large amounts (0.1 M) of fucose, (a methyl pentose) (6), probably relates to its similarity to o-methylglucopyranoside and that in large concentrations it probably blocks the binding site for this carbohydrate, thereby inhibiting MIF activity in vitro. Electrophoretically homogeneous MIF possessed MAF activity, or presumably the two activities proceed from the one chemical species.

The major difficulty and problem is that a wide variety of tissues have been found to have biological activity identical to MIF (7). The relevance, then, of our molecular parameters and findings for calf thymus MIF to human lymphokine is not established. Therefore, we are in the process of preparing more electrophoretically homogeneous MIF and developing antisera to this material using Freund's Complete Adjuvant. It would be hoped that such antisera would be demonstrated to be potent inhibitors of immunologically produced MIF activity from lymphocytes in vitro. I do not think that thymus MIF is a cytokine (7), as have been claimed to exist previously and hope to be able to demonstrate this by studies using antisera. Further Ochterlony double diffusion experiments will be performed to document the homogeneity of the purified MIF.

2. Lymphotactin

It has been clearly established that supernatants of transformed lymphocytes contain a material chemotactic for other lymphocytes (8) in vitro and in vivo. Further, degradation products of complement activation or protein hydrolysis by enzymes which are chemotactic for macrophages or PMN are not chemotactic for lymphocytes. We therefore took our thymus extracts and firstly demonstrated they contained lymphocyte chemotactic activity in vitro using Boyden chambers (9) and secondly, purified the material to apparent electrophoretic homogeneity (10). Lymphotactin (as we choose to call this specific chemotactic material) has a molecular weight of 10,500 daltons, an isoelectric point of 5.8 is thermo stable and is destroyed by trypsin but not by neuraminidase treatment.

We propose that this material which is released from transforming lymphocytes is a specific chemotactic signal to bring other "naive" lymphocytes into the area and into propinquity with the antigen. In this fashion the immunological defense system will be amplified by recruitment of new lymphocytes.

3. Permeability Factor

Over a decade ago extracts of lymphocytes and lymphoid tissue were shown to increase the permeability of the microcirculation (i.e., blueing reaction) in rat skin (11). This lymph node permeability factor (LNPF) was the first demonstration of a vascular response to lymphocytes in vivo. Approximately five years later supernatants from transformed lymphocytes were shown to contain a Skin Reactive Factor which would cause induration in rabbit or guinea pig skin

(12,13). For psychological reasons of marvelous subtlety, Skin Reactive Factor (SRF) and LNPF were not regarded as identical or even similar! LNPF does not cause induration in rat skin; the rat's lymphatic drainage of his skin, however, is so profound that it is impossible to blister or cause induration in this animal. However, Skin Reactive Factor will cause blueing in the skin of the rat.

We have isolated and purified Skin Reactive Factor from transformed lymphocytes (PHA-stimulated rabbit lymphocytes in culture) and found it to have a molecular weight of approximately 84,000 daltons with an isoelectric point of 4.2 (14,15). Permeability Factor is also found in the lysosomes of lymphoblasts; lymphocytes essentially have no lysosomes, whereas lymphoblasts develop them in large amounts in the various vacuoles so characteristic of transformed lymphocytes. Again, SRF (induration of guinea pig skin) and LNPF (blueing reaction of rat skin) both have an isoelectric point of 4.2 and a molecular weight of 84,000. Both were purified to homogeneity in acrylamide gel electrophoresis (15) and both share the extremely interesting property of being completely inhibited by 10^{-5} M pepstatin! Both factors are also inhibited by pretreatment of the animals with antihistamines. Pepstatin, which is normally considered to be a specific inhibitor of cathepsin E and D (and possibly other acidic proteases) may be inhibiting the serine esterases on the surface of the mast cell which are essential to degranulation, or the purified permeability factor may be significantly altered by acid proteases in the skin itself.

4. Lung Permeability Factor

Our studies on the mechanism of the action of LNPF/SRF Permeability Factor has been rendered more interesting by our demonstration that an identical molecule exists in lung tissue extracts from a number of species. It is found in the lysosomal fraction of the lung and also can be found in macrophages obtained by pulmonary washout. Thus, it would appear that both lymphoblasts and macrophage lysosomes, and possibly others, contain this acidic permeability factor. The lung factor was also inhibited by pepstatin and benzamidine, another inhibitor of proteases. This lung factor was also inhibited in its action by antihistamines. Thus, we have started to speculate on the mechanism of pulmonary edema, particularly the "wet lung" often found in shock patients, particularly after trauma and gunshot wounds. Although it is considered that mast cells do not exist in the alveolar portion of the lung, clearly microscopic and ultrastructural analysis of the human and rodent lung indicate a small number of mast cells can be found even in the most remote portion of the alveolar tree. We propose the following mechanism: a) as a result of either profoundly increased phagocytosis due to infection, or lysosomal granule discharge due to endotoxin, that macrophages release their lysosomal contents into the extracellular space. b) Among those lysosomal enzymes which are often "drooled" during phagocytosis by macrophages, at some critical concentration is found the Permeability Factor described above

which will effect the degranulation of the few mast cells in the immediate area. c) As the result of the release of histamine *in situ*, the area around the macrophages becomes edematous with the extravasation of a number of macromolecules from within the circulation including most particularly complement. d) This local concentration of complement, which becomes rapidly activated, markedly assists the macrophages in dealing with infection. The "wet lung" response to shock or trauma is probably an aberrant one, since normal selection mechanisms over thousands of years would develop this system to defend the host against the most common challenge (namely, bacteria). e) Thus, it might be possible to treat with antihistamines patients who are at risk for pulmonary edema. We are currently exploring the possibility of experimentally creating pulmonary edema in the lung of rats. The major difficulty to date is firstly that the lymphatic drainage of rat (and rodent in general) lung is so extraordinary as to render it almost impossible to change the wet/dry ratio of lung tissue except under the most extreme challenges, and secondly, the administration of the purified Pulmonary Permeability Factor is rendered difficult by the fact that it is inhibited by serum. Interestingly enough, it is not, however, inhibited by plasma, which suggests that either the inhibitor is contained in the platelets and is released when the platelets degranulate during clotting or, like some of the blood clotting enzymes, are inhibited by the breakdown products peptide A and B from the conversion of fibrinogen to fibrin.

5. Macrophage Growth Factor

About eight years ago the concept that macrophages were post-mitotic cells produced by the maturation of stem cells was largely disproved, particularly for pulmonary-derived macrophages (see ref. 16) by a demonstration that the products of the cultivation of lung cells *in vitro* (used or conditioned medium) would, when mixed with F-12 medium and 15% fetal calf serum, allow the cultivation of mouse macrophages *in vitro*. This mitogenic macrophage growth factor from lung cells has been purified to electrophoretic homogeneity by us recently. All of the mitogenic activity that could be demonstrated in the used medium from the cultivation of mouse lung cells *in vitro*, was concentrated over 30,000 dalton ultrafilter and, at pH 4.2 by isoelectric focussing. Acrylamide gel electrophoresis of the middle of this pH 4.2 isoelectric focussing peak at 100 µg/gel indicated that approximately 80-85% of the protein in this fraction have electrolytic mobility of beta globulin; the rest of the material giving a series of bands spaced at decreasing distances behind this fraction. At pH 9.5 or pH 7. the pattern was essentially the same electrophoretically. When these gels were run with only 5 to 20 µg of protein and stained with Coomassie Blue in the usual technique, however, only one electrophoretic band could be clearly demonstrated. Further, if this isoelectric-focussed fraction was subjected to SDS electrophoresis at pH 7 in acrylamide gel in the usual fashion, only

one band could be visualized - each at 100 µg/gel with a molecular weight of 68,000 daltons. Finally, the fastest band of acrylamide gel electrophoresis containing the bulk of the protein and probably with a molecular weight of 68,000, was collected mechanically by elution from a series of analytical acrylamide gels and re-electrophoresed in the same fashion. Once again, the same pattern of increasing molecular weight sizes in the acrylamide gels could be visualized. The fact of this SDS homogeneity vs the apparent heterogeneity in the regular acrylamide gel electrophoresis, plus the re-establishment of a similar pattern in a regular electrophoresis of what should be an electrophoretically homogeneous band, suggests that this material is an equilibrium with its own aggregation products with each of the four smaller bands, which are less mobile in electrophoresis, representing multiples of the basic 68,000 dalton monomer. At a pH of 2.1 almost all of the factor is aggregated to large molecular weight complexes with a minority of the total protein remaining at 68,000 daltons. Since Macrophage Growth Factor is clearly related to Colony Stimulating Factor, this aggregation phenomenon is of considerable interest.

Finally, purified Macrophage Growth Factor is a potent inhibitor of trypsin, but not of either chymotrypsin or of elastase. This finding of a mitogenic growth factor functioning as a specific inhibitor of proteases is quite remarkable in view of the generally widespread feeling that proteolysis promotes proliferation and the demonstration that most protein inhibitors inhibit, not stimulate, mitotic activities. From the biochemical point of view the aggregability of purified Macrophage Growth Factor, which would not be demonstrable in acrylamide gel electrophoresis using the smaller amounts of material currently favored by practitioners of the art of acrylamide gel electrophoresis, is extremely interesting and its potent inhibitory property towards trypsin is most remarkable.

Conclusions:

I'm in the process of concluding with a reasonable degree of success our attempts to isolate and purify important lymphokines from thymus which are involved in the mediation of the inflammatory response (i.e., MIF, lymphotactin and LNPF/SRF). The remaining and most critical experiments are to create antisera to these thymus-derived lymphokine activities and then to demonstrate the ability of such antisera to inhibit lymphokine biological activity from the supernatants from transformed lymphocytes in vitro.

The finding of a hitherto unrecognized potent Pulmonary Permeability Factor which apparently functions via the degranulation of mast cells with concominant release of histamine, may have considerable clinical importance and suggest a new therapeutic modality for the control of pulmonary edema. The purification of Macrophage Growth Factor and its remarkable anti-trypsin property (circulating anti-trypsins of all kinds also inhibit chymotrypsin and elastase and are thereby clearly differentiated from this Pulmonary Permeability Factor) is of potentially great interest both in terms of biochemistry and too our understanding of the control of mitosis in vitro and in vivo.

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We have been able to isolate, purify and physicochemically characterize three important lymphokines; MIF, the chemotactic stimulant to lymphocytes, (lymphotactin) and Skin Reactive Factor/LNPF. Each of these molecules has been purified to electrophoretic homogeneity at least two different pH in acrylamide gel electrophoresis. The carbohydrate composition of MIF was determined and found to involve only two moieties, sialic acid and neutral sugar which may be O-methylglucopyranoside. Antisera against thymus-derived

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20. (continued)

MIF is currently being prepared. MIF has been shown to be inhibited by amino-valeric acid. Lymphotactin is a much smaller molecule (10,500) which is specifically chemotactic for lymphocytes. SRF/LNPF is a highly anionic protein with a molecular weight of approximately 84,000 and an isoelectric point of 4.2. This material increases the permeability of the microcirculation of the skin by the degranulation of mast cells and the release of histamine (i.e., it can be blocked by antihistamines in vivo). It is also inhibited by the acid protease inhibitor pepstatin.

We are currently involved in the purification of Macrophage Growth Factor released by lung cells in vitro into the medium, which has a molecular weight of 68,000 daltons and an isoelectric point of 4.2. This material aggretates with itself to form multiples of this sized monomer and possesses Colony Stimulating activity in vitro as well as Macrophage Growth Factor activity. It will also inhibit trypsin (but not chymotrypsin, elastase or sulfhydryl proteases).

All these materials are important to the immunologically mediated inflammatory process in general and to inflammation and edema in the lung in particular.